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Regulation of Focal Adhesion Kinase by a Novel Protein Inhibitor FIP200

Smita Abbi, Hiroki Ueda, Chuanhai Zheng, Lee Ann Cooper, Jihe Zhao, Renee Christopher, and Jun-Lin Guan*

Department of Molecular Medicine, College of Veterinary Medicine, Cornell University, Ithaca, New York 14853

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Focal adhesion kinase (FAK) is a major mediator of integrin signaling pathways. The mechanisms of regulation of FAK activity and its associated cellular functions are not very well understood. Here, we present data suggesting that a novel protein FIP200 functions as an inhibitor for FAK. We show the association of endogenous FIP200 with FAK, which is decreased upon integrin-mediated cell adhesion concomitant with FAK activation. In vitro- and in vivo-binding studies indicate that FIP200 interacts with FAK through multiple domains directly. FIP200 bound to the kinase domain of FAK inhibited its kinase activity in vitro and its autophosphorylation in vivo. Overexpression of FIP200 or its segments inhibited cell spreading, cell migration, and cell cycle progression, which correlated with their inhibition of FAK activity in vivo. The inhibition of these cellular functions by FIP200 could be rescued by coexpression of FAK. Last, we show that disruption of the functional interaction between endogenous FIP200 with FAK leads to increased FAK phosphorylation and partial restoration of cell cycle progression in cells plated on poly-L-lysine, providing further support for FIP200 as a negative regulator of FAK. Together, these results identify FIP200 as a novel protein inhibitor for FAK.

INTRODUCTION

Focal adhesion kinase (FAK) is a major mediator of signal transduction by integrins, which has been implicated in the regulation of cell spreading, migration, survival, and proliferation (Clark and Brugge, 1995; Schwartz *et al.*, 1995; Parsons, 1996; Cary and Guan, 1999; Schlaepfer *et al.*, 1999). FAK activation and tyrosine phosphorylation have been shown in a variety of cell types to be dependent on integrins binding to their extracellular ligands (Schwartz *et al.*, 1995). On its activation, FAK is autophosphorylated at Y397, which mediates FAK association with a number of Src homology 2 (SH2) domain-containing signaling molecules, including Src family kinases (Chan *et al.*, 1994; Cobb *et al.*, 1994; Schaller *et al.*, 1994; Xing *et al.*, 1994), p85 subunit of PI3K (Chen and Guan, 1994), phospholipase C-γ (Zhang *et al.*, 1999), and Grb7 (Han and Guan, 1999). FAK binding to Src family kinases has been proposed to allow phosphorylation of Y925 of FAK by Src, which binds to the SH2 domain of Grb2 (Schlaepfer *et al.*, 1994). The FAK/Src complex formation also leads to tyrosine phosphorylation of a number of other substrates, including paxillin (Burridge *et al.*, 1992; Schaller and Parsons, 1995), p130cas (Vuori *et al.*, 1996; Tachibana *et al.*, 1997), and Shc (Schlaepfer *et al.*, 1998). Recent studies have shown that Grb7 is phosphorylated by FAK in a Src-independent manner (Han *et al.*, 2000).

FAK and its downstream signaling pathways have been shown to play important roles in the regulation of cell spreading and migration (Ilic *et al.*, 1995; Cary *et al.*, 1996; Gilmore and Romer, 1996; Richardson and Parsons, 1996). $FAK^{-/-}$ fibroblasts derived from FAK-knockout mouse embryo showed a significant decrease in cell migration compared with the cells from wild-type mice (Ilic *et al.*, 1995). Similarly, inhibition of FAK by the FAK C-terminal recombinant protein (i.e., FRNK) caused decreased motility of both fibroblasts and endothelial cells (Gilmore and Romer, 1996), as well as a reduced rate of fibroblast spreading (Richardson and Parsons, 1996). Last, overexpression of FAK in a number of cell lines, including the $FAK^{-/-}$ cells, promoted their migration on fibronectins (FN) (Cary *et al.*, 1996; Owen *et al.*, 1999; Sieg *et al.*, 1999). FAK signaling pathways have also

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* Corresponding author. E-mail address: jg19@cornell.edu.

Abbreviations used: BrdU, bromodeoxyuridine; CT-FIP, C-terminal FIP; FAK, focal adhesion kinase; FIP200, FAK-family interacting protein of 200 kDa; FN, XXXX; GFP, green fluorescent protein; GST, glutathione *S*-transferase; HA, hemagglutinin; Ig, immunoglobulin; mAb, monoclonal antibody; MD-FIP, middle domain FIP; NT-FIP, N-terminal FIP; PCR, polymerase chain reaction; PLL, poly-L-lysine; SH2, Src homology 2.

been shown to regulate cell survival and cell cycle progression in integrin-mediated cell adhesion. Overexpression of FAK protected cells from apoptosis induced by cell detachment, serum withdraw, or other treatments in MDCK cells or primary fibroblasts (Frisch *et al.*, 1996; Ilic *et al.*, 1998; Chan *et al.*, 1999). Conversely, inhibition of FAK by treatment of tumor cell lines with FAK antisense oligonucleotides (Xu *et al.*, 1996) or by microinjection of CEF cells with an anti-FAK monoclonal antibody- (mAb; Hungerford *et al.*, 1996) induced apoptosis. Microinjection of the C-terminal fragment of FAK into either fibroblasts or endothelial cells inhibited cell cycle progression as measured by bromodeoxyuridine (BrdU) incorporation (Gilmore and Romer, 1996). Inhibition of FAK tyrosine phosphorylation by disruption of FN matrix assembly also resulted in the delay of the G1 to S transition, suggesting a role for FAK in cell cycle progression (Sechler and Schwarzbauer, 1998). Finally, using a tetracycline-regulated expression system, we have shown recently that expression of wild-type FAK accelerated G1 to S transition, whereas expression of a dominant negative FAK mutant inhibited cell cycle progression at G1 phase (Zhao *et al.*, 1998).

In contrast to rapid progress in elucidating the FAK downstream signaling pathways, relatively little is known about the mechanisms of regulation of FAK activity and its associated cellular functions. Using the yeast two-hybrid screen, we have recently identified a novel protein, FAK-family interacting protein of 200 kDa (FIP200), that is associated with the FAK-related tyrosine kinase Pyk2 (Ueda *et al.*, 2000). Our initial analysis indicated that FIP200 could inhibit the kinase and cellular activity of Pyk2 by binding to its kinase domain directly. Furthermore, FIP200 could also bind to FAK. Interestingly, both FIP200 and FAK are widely expressed in a variety of tissues and cell lines in contrast to the limited expression pattern of Pyk2 (Avraham *et al.*, 1995; Lev *et al.*, 1995; Schwartz *et al.*, 1995; Nagase *et al.*, 1996; Ueda *et al.*, 2000). This suggests a potentially important role for FIP200's interaction with FAK in some fundamental cellular functions. In this report, we show that FIP200 could also bind to the kinase domain of FAK and function as a protein inhibitor for FAK kinase activity and its associated cellular functions.

MATERIALS AND METHODS

Antibodies

Polyclonal antibodies against the C-terminal FIP200 (residues 1374–1591; anti-FIP200C; Ueda *et al.*, 2000), rabbit antiserum against FAK (Chen and Guan, 1994), mouse mAb KT3 (Cary *et al.*, 1996), and mouse mAb 12CA5 that recognize the hemagglutinin (HA) epitope tag (Chen *et al.*, 1995) have been described previously. Antiserum against the N-terminal segment of FIP200 was prepared in rabbits using a glutathione *S*-transferase (GST)-fusion protein containing residues 1–373 within N terminus of FIP200. Anti-FIP200N antibodies were then affinity purified from the antiserum using the same fusion protein immobilized on glutathione-Sepharose as an affinity matrix. Mouse mAbs against FAK, Pyk2, and paxillin, and antiphosphotyrosine antibody, PY20, were purchased from Transduction Laboratories (Lexington, KY). Rabbit antibody against phosphorylated Y397 of FAK (anti-pFAKY397) was purchased from Biosource (Camarillo, CA). Rabbit anti-HA (HA probe), mouse mAb against Myc epitope tag (9E10), and rabbit polyclonal anti-green fluorescent protein (GFP) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-β-Gal was from 5 prime- 3 prime, Inc. (Boulder, CO). Mouse monoclonal anti-Flag, anti-BrdU, fluorescein-conjugated goat anti-rabbit immunoglobulin (Ig) G, and rhodamine-conjugated goat anti-mouse IgG were purchased from Sigma (St. Louis, MO).

Construction of Expression Vectors

The expression vectors pSG5-FIP200, pSG5-N-terminal-FIP (NT-FIP), and pSG5-C-terminal-FIP (CT-FIP) encoding Flag-tagged full-length NT-FIP and CT-FIP have been described previously (Ueda *et al.*, 2000). pSG5-middle domain-FIP (MD-FIP) encoding Flag-tagged middle domain of FIP200 was generated by amplifying residues 639-1373 of FIP200 using primers with *Eco*RV site at the 5′ end and *Bgl*Isite at the 3′ site. The region was subsequently cloned into the corresponding cloning sites in pSG5 vector. Similarly, expression vectors pKH3-FIP200, pKH3-NT-FIP, pKH3-MD-FIP, and pKH3-CT-FIP encoding HA-tagged FIP200 or fragments were generated by amplifying residues 1–1591, 1–638, 639-1373, and 1374–1591 with the addition of *Sma*I site at 5′ end and *Eco*RV site at 3′ end. These fragments were subsequently digested and cloned into corresponding cloning sites in pKH3 vector.

pGEX-CT-FIP has been described previously (Ueda *et al.*, 2000). pGEX-NT-FIP was constructed by performing polymerase chain reaction (PCR) to generate a 1.1-kb N-terminal fragment corresponding to residues 1–373 within NT-FIP with the addition of *Sma*I site at the 5′ end and *Eco*RV site at the 3′ end. This fragment was digested with *Sma*I and *Eco*RV and was inserted into the corresponding cloning site of pGEX-2T vector. pGEX-MD-FIP was generated by amplifying region corresponding to residue 639-1373 of plasmid encoding full-length-FIP200. The primers included a *Sma*I site at 5′ end and *Eco*RV site at 3′ end. The fragment was digested with *Sma*I and *Eco*RV and was inserted into corresponding sites into the pGEX-2T vector.

FAK segment containing N-terminal domain (NT-FAK) was generated by PCR amplification using the forward (5′-CTGGATCCATGGCAGCTTACCTTG-3′) and reverse (5′-ATGATATCTTAAGTATCTTC TTCATC-3′) primers. The PCR product was digested with *Bam*HI and *Eco*RV and was cloned into pKH3 at *Bam*HI and *Sma*I site to generate pKH3-NT-FAK. FAK segment containing the kinase domain (KD-FAK) was generated by PCR amplification using the forward (5′-ATGATATCAACCAGAGATTATGAAATTC-3′) and reverse (5′-GCTTTAAATTAAGTAAACCTGGGTCGTC-TAC-3′) primers. The PCR product was digested with *Eco*RV and *Dra*I and was cloned into pKH3 at *Sma*I site to generate pKH3-KD-FAK. The same primers were used to amplify the kinase domain with K454 to R mutation using a FAK cDNA with this mutation as the template (Cary *et al.*, 1996). This fragment was then cloned into pKH3 vector to make the HA-tagged KD^{KR} construct. The expression vectors encoding full-length HA-tagged FAK and the C-terminal FAK have been described previously (Zhao *et al.*, 1998).

The kinase domain of Pyk2 was generated by PCR amplification using the sense (5′-CCAGGATCCGGCATTGCCCGTGAAGATG-3′) and antisense (5′-ATGAATTCGCTTCACACCAGCTCGGTG-3′) oligonucleotides. The product was then inserted into pKH3 to generate pKH3-KD-Pyk2. The vector encoding full-length Pyk2 has been described previously (Zheng *et al.*, 1998). The expression vectors encoding HA-tagged Grb7 and the control protein (Grb7-SH2 domain) have been described previously (Han and Guan, 1999). Expression vectors encoding GFP-paxillin, HA-Shc, and Myc-p130cas were kind gifts from Drs. C. Turner (Upstate Medical Center, Syracuse, NY), D. Schlaepfer (Scripps Research Institute, La Jolla, CA) and S. Hanks (Vanderbilt University, TN), respectively.

In Vitro Binding

GST fusion proteins were produced and purified using a protease-defective *Escherichia coli* strain BL21-Dex, as described previously (Ueda *et al.*, 2000). GST fusion proteins (3 μg) were immobilized on

glutathione-agarose beads and were then incubated for 4 h at 4°C with lysates (200 μg) prepared from 293 cells that had been transfected with expression vectors encoding kinase domain of Pyk2, HA-FAK, or its fragments. After washing, the bound proteins were analyzed by Western blotting with anti-HA (1:2000) as described below. For binding to the recombinant FAK, His-tagged recombinant FAK was purified from baculovirus-infected sf21 cells as described previously (Withers *et al.*, 1996). GST-fusion proteins (5 μg) were equalized for amount of glutathione agarose beads and were incubated with 1 μg of purified His-tagged FAK in binding buffer (20 mM Tris, pH 8.0, 137 mM NaCl, 1 mM $MgCl_2$, and 1% Triton) overnight at 4°C with rotation. The samples were then washed five times with binding buffer, boiled in SDS buffer, resolved by SDS-PAGE, and western blotted with α-FAK antibody.

Immunoprecipitation and Western Blot

For most experiments, cells were lysed with 1% NP-40 lysis buffer (20 mM Tris, pH 8.0, 137 mM NaCl, 1% Nonidet P40, 10% glycerol, 1 mM Na_3VO_4, 1 mM phenyl methyl sulfoxide, 10 μg/ml aprotinin, and 20 μg/ml leupeptin). For experiments to detect phosphorylation of HA-Shc, cells were lysed in the modified RIPA lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.3% sodium deoxycholate, 0.1% Nonidet P-40, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EDTA, 0.2 mM EGTA, 20 mM NaF, 25 μM $ZnCl_2$, 1 mM NaVO4, 1 mM phenyl methyl sulfoxide, 10 μg/ml aprotinin, and 2 μg/ml leupeptin) as described previously (Zhao *et al.*, 1998). Immunoprecipitation was carried out at 4°C by incubating cell lysates for 2–4 h with indicated antibodies followed by incubation for 1 h with Protein A-Sepharose or Protein G-Plus. Immunoprecipitates were washed three times in lysis buffer without protease inhibitors. The beads were then resuspended in SDS-PAGE sample buffer, boiled for 5 min, and resolved by SDS-PAGE. Western blotting was performed with appropriate antibodies as indicated, using the Amersham enhanced chemiluminescent system (Arlington Heights, IL), as described previously (Chen *et al.*, 1995; Ueda *et al.*, 2000). In some experiments, whole cell lysates were analyzed directly by Western blotting.

FAK In Vitro Kinase Assay

FAK was immunoprecipitated from Chinese hamster ovary cells overexpressing FAK (Cary *et al.*, 1996). Aliquots of the immune complex were assayed for kinase activity as described previously (Ueda *et al.*, 2000) in the presence of various amounts of GST fusion proteins containing FIP200 segments or GST alone.

Measurement of Cell Spreading

NIH3T3 cells were transfected using the LipofectAmine and PLUS transfection reagents (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. One day after transfection, the cells were replated on FN (10 μg/ml), fixed in formaldehyde, and processed for immunofluorescence staining (see below). Alternatively, cells were cotransfected with a plasmid encoding β-gal along with the indicated vectors. One day after transfection, cells were replated on FN (10 μg/ml) for 30 min, fixed, and assayed for β-gal activity as described previously (Cary *et al.*, 1996). At least 60 positively transfected cells (blue) were counted for their spreading phenotype in each transfection in three independent experiments.

Cell Migration Assays

NIH 3T3 cells were cotransfected with various vectors along with a plasmid encoding GFP in 7:1 ratio using the LipofectAmine and PLUS transfection reagents (Life Technologies) according to the manufacturer's instructions. One or 2 d after transfection, the cell monolayer was wounded with a p10 tip. The plates were then washed and incubated at 37°C in growth medium for 8 h. Phase contrast and fluorescence images were taken every 2 h until the wound closed (~10 h). The rate of migration was calculated by measuring the distance moved toward the center of the wound in 8 h. Motility assays using OMAware were as described previously (Han *et al.*, 2000).

Measurement of Cell Cycle Progression by BrdU Incorporation

BrdU incorporation assays were performed as described previously (Zhao *et al.*, 1998). Briefly, NIH 3T3 cells were transfected using the LipofectAmine and PLUS transfection reagents (Life Technologies) according to the manufacturer's instructions. The subconfluent transfected cells were serum starved for 24 h in DME with 0.5% CS. They were then replated on FN (10 μg/ml) and incubated for 16 h with 100 μM BrdU (Sigma) in DME plus 10% CS. For experiments with FAK-KD^{KR} mutant, cells were serum starved for 30 h in 0.5% serum. They were then replated on FN (10 μg/ml) or poly-L-lysine (PLL; 0.1 mg/ml) and incubated for 20 h with 100 μM BrdU in 1% serum. Cellular DNA was digested with 0.5 U/μl DNaseI (New England Biolabs, Beverly, MA) for 30 min at 37°C. Cells were then processed for double immunofluorescence staining with polyclonal anti-HA (HA probe; 1:300) and monoclonal anti-BrdU (1:300) as described below. At least 80 positively transfected cells (as recognized by anti-HA) in multiple fields were scored for BrdU staining in each independent experiment. For FAK rescue experiments, an expression plasmid encoding β-Gal was also included in transfections. Cells were then analyzed for BrdU incorporation as described above, except that the positively transfected cells were identified by immunostaining with polyclonal anti-β-Gal. The percentage of $BrdU^+/\beta\text{-}Gal^+$ cells was determined by analyzing 40–50 $\beta\text{-}Gal^+$ cells for each transfection in multiple fields.

Immunofluorescence Staining

Cells were processed for immunofluorescence staining as described previously (Zhao *et al.*, 1998). The primary antibodies used were polyclonal anti-FIP200N (1:200), monoclonal anti-FAK (1:100), polyclonal anti-HA (1:200), polyclonal anti-β-Gal (1:300), monoclonal anti-BrdU (1:200), and monoclonal antivinculin (1:50). The secondary antibodies used were fluorescein-conjugated goat anti-rabbit IgG (1:300) and rhodamine-conjugated goat anti-mouse IgG (1:200). The cells were mounted on Slowfade (Molecular Probes, Eugene, OR) and examined. The image of stained cells was captured using an immunofluorescence microscope (Olympus, Tokyo, Japan) and a charged-coupled device camera.

RESULTS

Association of Endogenous FIP200 with FAK

To explore the mechanism and potential function of FIP200 interaction with FAK, we first analyzed interaction of endogenous FIP200 and FAK. Lysates were prepared from cells that had been suspended or replated on FN, type IV collagen, or type I collagen. They were immunoprecipitated by an antibody against FIP200 and then subjected to western blotting with anti-FAK to detect associated FAK in the immune complexes. Figure 1A shows association of endogenous FAK with FIP200 and that the association was decreased upon cell adhesion to FN, and to a less extent, type IV collagens or type I collagen. Western blotting of the immunoprecipitates with another antibody against FIP200 showed similar amounts of FIP200 precipitated from cells lysates under these different conditions (Figure 1B). Consistent with previous studies (Schwartz *et al.*, 1995), cell adhesion to FN, and to a less extent, type IV collagens or type I collagen, activated FAK that lead to increased FAK autophosphorylation at Y397 (Figure 1, C and D). These results

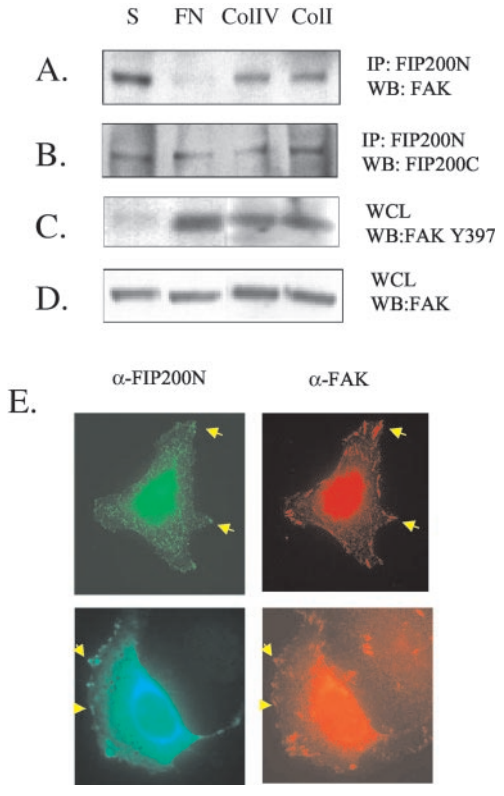

Figure 1. Association and localization of FIP200 and FAK. (A-D) Lysates were prepared from MDA-MB231 breast carcinoma cells that had been suspended, or replated on FN, type IV collagen, or type I collagen, as indicated. They were immunoprecipitated by anti-FIP200N and then analyzed by Western blotting with anti-FAK (A) or anti-FIP200C (B). The whole cell lysates (WCL) were also analyzed directly by Western blotting with anti-pFAKY397 (C) or anti-FAK (D). (E) NIH 3T3 cells (top panels) or ECV304 cells transfected with FIP200 (bottom panels) were processed for immunofluorescence as described in "Materials and Methods" using anti-FIP200N or anti-FAK, as indicated. Examples of colocalization of FIP200 and FAK in peripheral focal contacts are marked by arrows.

suggest that FIP200 dissociation from FAK is correlated with FAK activation during cell adhesion, which is consistent with our previous finding that FIP200 may also function as a protein inhibitor for FAK (Ueda *et al.*, 2000). These coimmunoprecipitation analyses also detected association of endogenous FIP200 and FAK in several other cell lines, including rat aortic smooth muscle cells, 293 cells, and NIH 3T3 cells (S. Abbi and J. Guan, unpublished data).

Our previous studies suggested that FIP200 was predominantly localized in the cytoplasm (Ueda *et al.*, 2000). Using the new polyclonal antibody against the N-terminal domain of FIP200, we detected presence of endogenous FIP200 in the focal contacts in the cell periphery in addition to the cytoplasmic staining in a fraction of the cells (Figure 1E). Costaining with anti-FAK (Figure 1E, top panels) or anti-vinculin (S. Abbi and J. Guan, unpublished data) showed partial colocalization of FIP200 with FAK and vinculin in the focal contacts. This partial colocalization of FIP200 with FAK in focal contacts in the periphery of the cells was also seen more clearly in cells transfected with the full-length FIP200 (Figure 1E, bottom panels). These results suggested that at least part of FIP200 was partially colocalized with FAK.

FIP200 Association with FAK through Multiple Interaction Domains

To define the FAK-binding domains within FIP200, we coexpressed HA-tagged FAK with Flag-tagged FIP200 and several FIP200 segments (see Figure 2A) in 293 cells. Immunoprecipitations were performed with anti-Flag antibody and were followed by western blotting with anti-FAK antibody. As shown in Figure 2B, FAK is coprecipitated with the full-length FIP200 and the CT-FIP, which is consistent with our previous result (Ueda *et al.*, 2000). Surprisingly, however, both the FIP200 NT-FIP and MD-FIP segments also associated with FAK in these experiments. We then performed in vitro-binding assays to determine whether all three FIP200 segments bound to the same region on FAK. Figure 2C shows that GST fusion proteins containing any of the three FIP200 segments bound to the full-length FAK, whereas GST alone did not. Interestingly, GST fusion proteins containing NT-FIP or MD-FIP bound to the kinase domain of FAK, whereas GST fusion protein containing CT-FIP bound to the N-terminal region of FAK. None of the GST fusion proteins bound to the C-terminal region of FAK. The interaction of NT-FIP and MD-FIP with the kinase domain of FAK was specific because they did not interact with the kinase domain of Pyk2, a homolog of FAK, in the same experiment. Likewise, GST alone did not bind to any of the FAK domains as expected. To examine whether all three FIP200 fragments bound to FAK directly or indirectly through other proteins in the 293 cell lysates, we used purified recombinant FAK from insect cells in the same in vitro-binding assays. Figure 2D shows that GST fusion proteins containing NT-FIP, MD-FIP, or CT-FIP, but not GST alone, bound to the recombinant FAK. Taken together, these results demonstrate that FIP200 could associate directly and specifically with FAK through multiple interaction domains.

FIP200 Inhibition of FAK Kinase Activity and Autophosphorylation

The binding of FIP200 to FAK kinase domain raised the possibility that FIP200 may have an effect on FAK kinase activity. To test this directly, we performed FAK in vitro kinase assays using E4Y1 as an exogenous substrate in the presence of different amounts of purified GST fusion protein containing the FIP200 segments or GST alone as a control. Figure 3A shows that the GST fusion proteins containing the NT-FIP and MD-FIP inhibited FAK kinase activity, whereas GST alone did not have any effect. GST fusion protein containing NT-FIP showed a significantly greater inhibitory effect than GST fusion protein containing CT-FIP. GST fusion protein containing MD-FIP showed an intermediary activity, which was also significantly higher than GST fusion protein containing CT-FIP. In particular, at lower concentrations (e.g., <5 pmol/reaction), GST fusion proteins containing NT-FIP or MD-FIP reduced FAK kinase activity, whereas CT-FIP did not, suggesting that NT-FIP and MD-FIP are more effective than CT-FIP in the inhibition of FAK kinase activity in vitro. These FIP200 segments also inhibited FAK from SYF cells (deficient in Src, Yes, and Fyn expres-

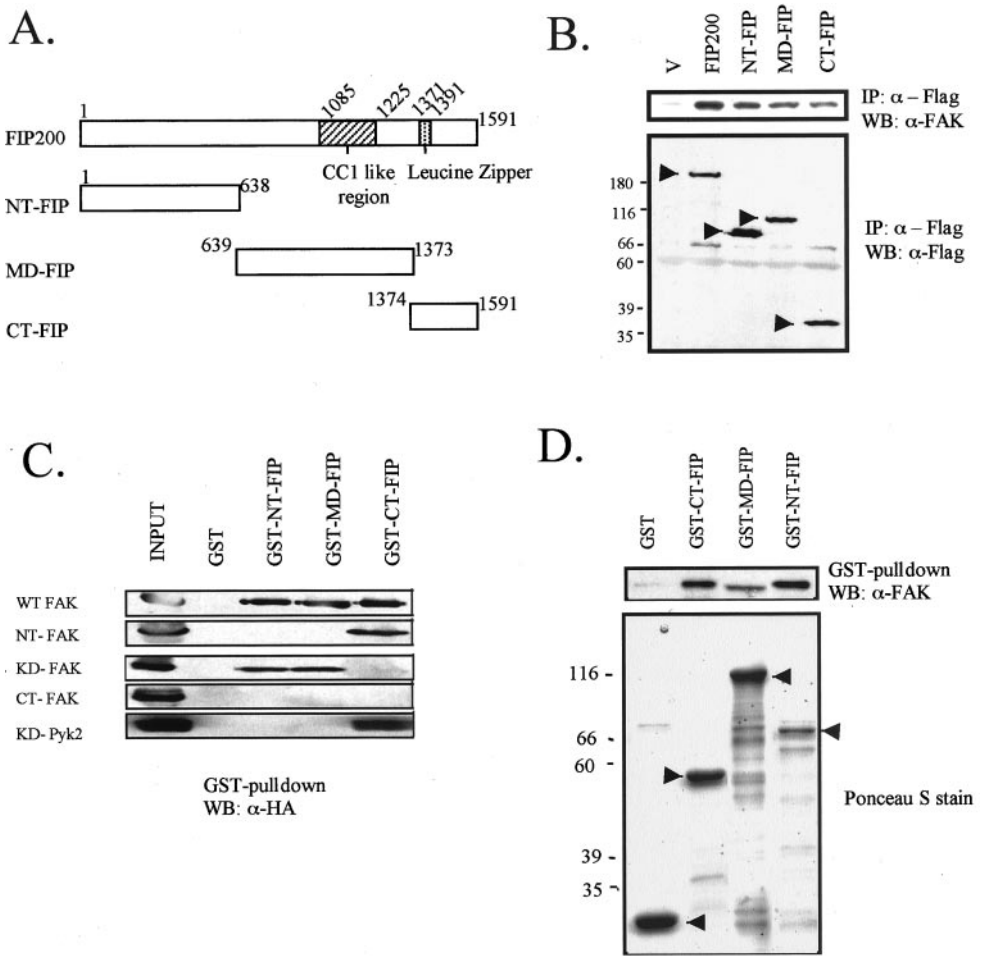

Figure 2. Analysis of FIP200 association with FAK. (A) A schematic of full-length FIP200 is shown on top. NT-FIP and CT-FIP segments, and MD-FIP of FIP200 are shown below. (B) 293T cells were transfected with an expression vector encoding HA-FAK and vectors encoding Flag-FIP200, its segments or empty vector control (V), as indicated. Lysates were immunoprecipitated with anti-Flag followed by Western blotting with anti-FAK to detect associated HA-FAK (top panel) or with anti-Flag to verify similar amounts of samples in the immunoprecipitates (bottom panel; Flag-FIP200 and segments are marked by arrowheads). (C) 293T cells were transfected with vectors encoding HA-FAK (WT) or its fragments (N-terminal, kinase domain and C-terminal) or with kinase domain of Pyk2, as indicated. Lysates from the transfected cells were then incubated with immobilized GST fusion proteins containing FIP200 segments or GST alone, as indicated. The bound proteins were resolved on SDS-PAGE and were analyzed by Western blotting with mAb 12CA5 (anti-HA). (D) Equal amounts of immobilized GST-fusion proteins containing FIP200 fragments, or GST alone, were incubated with 1 μg of recombinant FAK. The bound proteins were resolved on SDS-PAGE and were analyzed by Western blotting with anti-FAK (top panel). The membrane was also stained with Ponceau S stain to detect GST-fusion proteins (marked with arrowheads; bottom panel).

sion) to the same extent as FAK from wild-type control cells (S. Abbi and J. Guan, unpublished data), suggesting that FIP200 inhibited the kinase activity of FAK directly, but not through its potential effects on the associated Src family kinases.

We next examined the effect of FIP200 and its segments on cell adhesion-induced FAK phosphorylation in intact cells. As shown in Figure 3B (Top and middle panels), expression of FIP200 suppressed tyrosine phosphorylation of FAK after adhesion to FN (Figure 3B, compare lanes FIP200 and V). Expression of NT-FIP or MD-FIP also inhibited FAK phosphorylation (Figure 3B, compare lanes NT-FIP and MD-FIP with lane V), whereas CT-FIP did not have any effect (Figure 3B, compare lanes CT-FIP and V). Similar expression levels of FIP200 fragments were verified by western blotting with anti-HA (Figure 3B, lower panel). Together, these results indicate that binding of FIP200 to FAK through interactions at different domains could inhibit FAK kinase activity in vitro. However, they also suggest that the quantitative difference of the in vitro inhibitory activity of FIP200 segments could lead to a differential inhibition of FAK activity in intact cells by NT-FIP and MD-FIP, but not by CT-FIP.

Effects of FIP200 on FAK Downstream Signaling

Activation and autophosphorylation of FAK have been suggested to lead to tyrosine phosphorylation of several other cellular proteins, including paxillin, p130cas, Grb7, and Shc (Burridge *et al.*, 1992; Schaller and Parsons, 1995; Vuori *et al.*, 1996; Tachibana *et al.*, 1997; Schlaepfer *et al.*, 1998; Han *et al.*, 2000). Therefore, we examined the effects of FIP200 on the FAK-promoted activation of these downstream targets. Figure 4 shows that overexpression of FAK induced tyrosine phosphorylation of all four potential substrates, paxillin, p130cas, Grb7, and Shc, as observed previously (Burridge *et al.*, 1992; Schaller and Parsons, 1995; Vuori *et al.*, 1996; Tachibana *et al.*, 1997; Schlaepfer *et al.*, 1998; Han *et al.*, 2000). Interestingly, overexpression of NT-FIP, which had maximum inhibition of FAK activation and phosphorylation among the segments (see Figure 3), reduced cell adhesion-

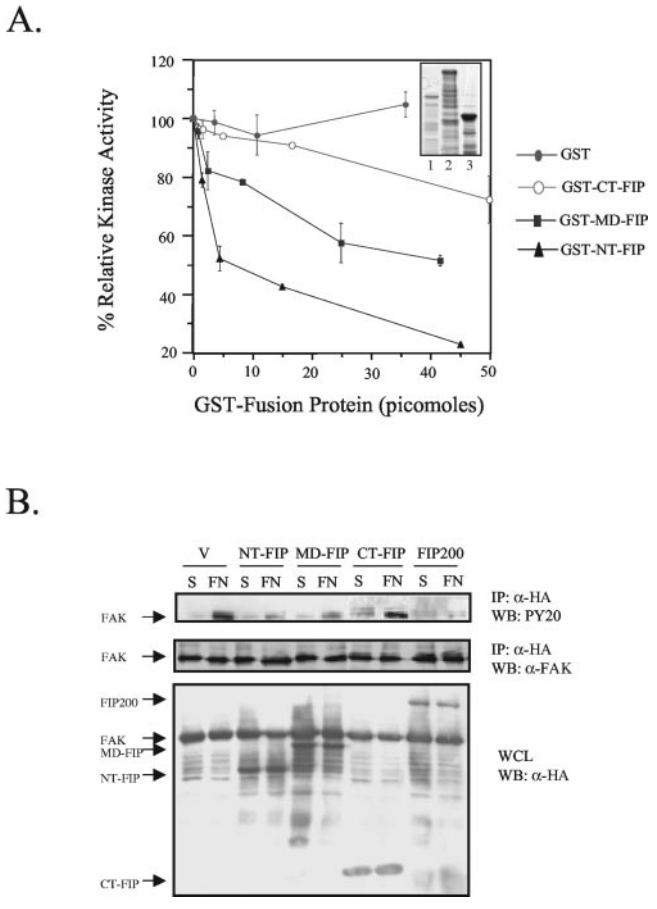

Figure 3. Inhibition of FAK activity by FIP200. (A) The kinase activity of FAK was assayed in the presence of various amounts of GST fusion proteins containing FIP200 segments, or GST alone, as indicated. Relative kinase activities were normalized to FAK activity in the absence of GST fusion protein. The mean ± SE of relative kinase activities from three independent experiments are shown. The inset shows Coomassie blue staining of a representation preparation of the fusion proteins (1, GST-NT-FIP; 2, GST-MD-FIP; and 3, GST-CT-FIP). (B) 293T cells were cotransfected with plasmid encoding HA-FAK and HA-FIP200, its segments, or vector control, as indicated. One day after transfection, cells were trypsinized and either kept in suspension (S) or replated on FN (10 μg/ml) for 30 min. They were then lysed and immunoprecipitated with anti-HA followed by Western blotting with PY20 to detect FAK phosphorylation (top panel) or anti-HA to verify similar FAK expression levels (middle panel). The corresponding whole cell lysates (WCL) were resolved on a SDS-PAGE gel and were western blotted with anti-HA to detect similar amounts of FIP200 and its fragments (marked by arrowheads, bottom panel).

dependent paxillin and Shc phosphorylation by FAK (Figure 3, A and C), but had little effect on p130cas and Grb7 phosphorylation (Figure 3, B and D). The mechanism of FIP200's selective inhibition of FAK downstream targets is unknown. It is possible that the threshold activity of FAK required for its phosphorylation of these substrates is different. Thus, inhibition of FAK by FIP200 under these experimental conditions could be sufficient to inhibit paxillin and Shc phosphorylation, but not for p130cas and Grb7 phosphorylation.

Effects of FIP200 on FAK-Dependent Cell Spreading and Migration

We next examined the effects of FIP200 on FAK-regulated cellular functions, including cell spreading and migration, and cell cycle progression. To study cell spreading, we transiently transfected NIH3T3 cells with the expression vectors encoding FIP200 or its fragments (see Figure 2A). The effects on cell spreading on FN were assessed initially by immunofluorescent staining with anti-HA antibody to mark the positively transfected cells, and with anti-vinculin antibodies to mark the background untransfected cells in the same field. Figure 5A shows that transfection of the cells with full-length FIP200 prevented cell spreading on FN (Figure 5, top panels), whereas expression of the CT-FIP did not affect cell spreading (Figure 5, bottom panels). Similar studies showed that expression of NT-FIP or MD-FIP also inhibited cell spreading (S. Abbi and J. Guan, unpublished data). The differences in cell spreading were still apparent 4 h after replating on FN (S. Abbi and J. Guan, unpublished data), although all cells were completely spread after overnight incubation (see Figure 7A), suggesting that inhibition of cell spreading by FIP200 was transient. We also used cotransfection of an expression vector encoding β-gal to identify the positively transfected cells in the cell spreading assays. Figure 5B shows similar results using this method. Expression of FIP-200, NT-FIP, or MD-FIP inhibited cell spreading by ~50% compared with control untransfected cells or cells expressing CT-FIP. The correlation of cell spreading inhibition by NT-FIP and MD-FIP, but not CT-FIP, with their inhibition of FAK activity (see Figure 3) suggested that FIP200 might inhibit cell spreading by its inhibition of endogenous FAK functions. Consistent with this possibility, coexpression of FAK with FIP200 rescued inhibition of cell spreading by FIP200 (compare the first and the last lane in Figure 5B), although overexpression of FAK alone had no effect on cell spreading under these conditions. Western blotting of aliquots of lysates from the transfected cells showed similar expression levels of FIP200 and its fragments and a lack of effects of FAK coexpression on the levels of FIP200 (Figure 5, C and D).

The effect of FIP200 and its fragments on cell migration was assessed by using monolayer-wounding assays after transient transfection of NIH3T3 cells with expression vectors encoding FIP200 or its fragments along with a plasmid encoding GFP. Phase contrast and fluorescence images were captured at regular intervals after wounding to monitor the movement of cells from the wound edge to the center of the wound. The rate of migration was then calculated for transfected cells at the edge of the wound by measuring the distance that the GFP-positive cells moved toward the center of the wound in 8 h. As shown in Figure 6A, cells transfected with the control vector (Figure 6V) moved toward the center of the wound at the same rate as the surrounding untransfected cells. In contrast, the FIP200-transfected cells moved much less than the surrounding untransfected cells. Quantification of the rate of migration showed that FIP200, NT-FIP, and MD-FIP inhibited cell migration by ~60–80%, whereas CT-FIP had no effect (Figure 6B). Furthermore, coexpression of FAK or paxillin with FIP200 rescued inhibi-

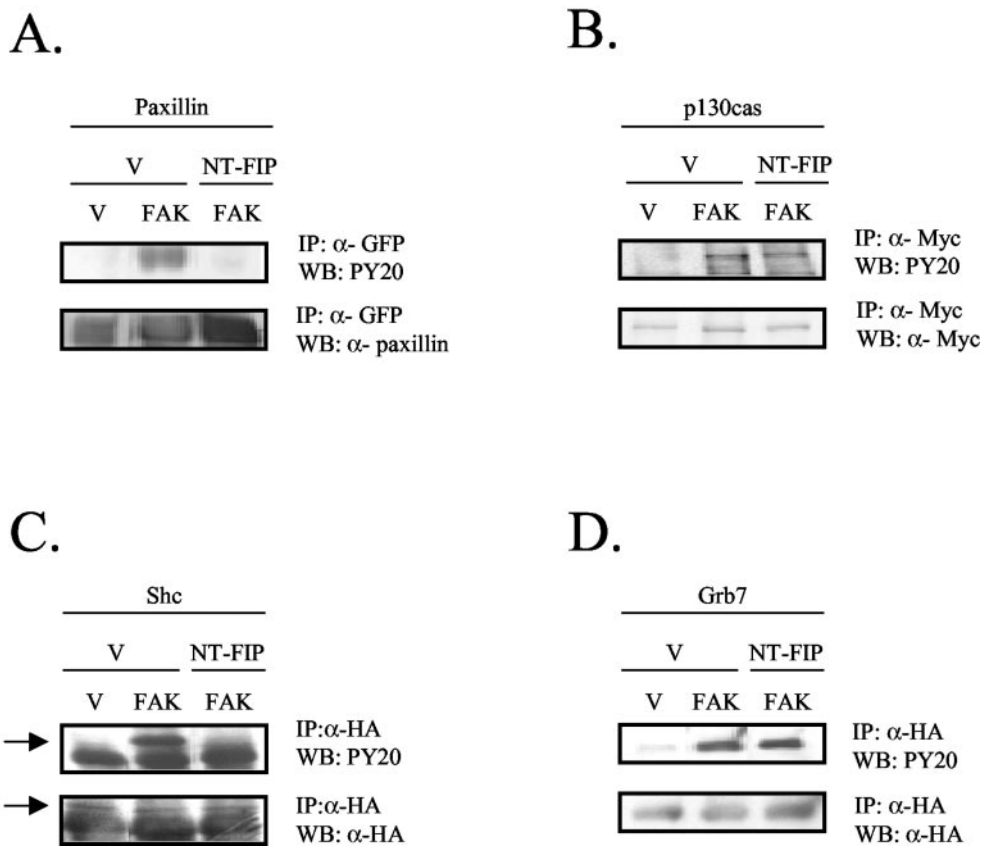

Figure 4. Effects of FIP200 on FAK downstream signaling. 293 cells were cotransfected with plasmids encoding FAK, NT-FIP, or empty vector as controls, along with vectors encoding GFP-paxillin (A), Myc-p130cas (B), HA-Shc (C), or HA-Grb7 (D), as indicated. One day after transfection, cells were trypsinized and replated on FN (10 μg/ml) for 30 min. Whole cell lysates were then immunoprecipitated with anti-GFP, anti-Myc, or anti-HA to pull-down epitope-tagged paxillin, p130cas, Shc, and Grb7, respectively. The immune complexes were then analyzed by Western blotting with PY20 to detect their phosphorylation (top panels), or with antipaxillin, anti-Myc, or anti-HA to show their respective expression levels (bottom panels). The position of HA-Shc is indicated by arrows in C.

tion of cell migration by FIP200 (Figure 6B). Similar results were also obtained using an alternative cell migration assays employing a time-lapse imaging-based computerized motility analysis method OMAware, as described previously (Han *et al*., 2000). Although FIP200 inhibited cell migration, coexpression with FAK reversed this inhibition to control levels (Figure 6C). Taken together, these results demonstrate that FIP200 inhibition of FAK leads to inhibition of FAK-dependent cell spreading and migration and suggest that inhibition of paxillin phosphorylation downstream of FAK might be responsible for these effects.

FIP200 Inhibition of Cell Proliferation and Its Rescue by FAK

To explore a potential role for FIP200 in cell cycle progression, we transiently transfected NIH3T3 cells with the expression vectors encoding FIP200 or its fragments (see Figure 2A), and then measured the extent of BrdU incorporation. Figure 7A shows that overexpression of FIP200 inhibited cell cycle progression as measured by BrdU incorporation (Figure 7A, top panels). Expression of a control vector encoding an irrelevant protein did not affect BrdU incorporation under the same conditions (Figure 7A, bottom panels). Quantitative analysis indicated that FIP200 inhibited cell cycle progression by ~90% compared with cells transfected with the control plasmid or mock-transfected cells (Figure 7B). Similar analysis showed that NT-FIP and MD-FIP also inhibited BrdU incorporation to a similar extent as the full-length FIP200, whereas CT-FIP did not have any effect. There was no evidence of apoptosis in any of the transfected cells (S. Abbi, H. Ueda, and J. Guan, unpublished data), suggesting that the cell cycle effects are not due to possible role of FIP200 or its fragments in cell survival or apoptosis.

FAK has been shown to play a role in cell cycle progression (Zhao *et al*., 1998), and we have shown that FIP200 can inhibit FAK activity. Therefore, we examined if overexpression of FAK along with FIP200 could rescue this inhibition of cell cycle progression. FAK alone did not promote cell proliferation under these conditions, but it rescued the inhibition of BrdU incorporation by FIP200 to the control levels (Figure 7C). Western blotting of aliquots of cell lysates showed that coexpression of FAK did not affect the expression levels of FIP200 (Figure 7C, inset). Together, these data indicate that FIP200 inhibition of FAK also leads to inhibition of FAK-dependent cell cycle progression.

Effects of Disruption of Functional Interaction between Endogenous FIP200 and FAK

We also investigated the role of FIP200 as a protein inhibitor for FAK by disrupting the functional interaction of these two proteins. Although FIP200 can associate with FAK through more than one domains (see Figure 2), FIP200 binding to FAK kinase domain is responsible for its inhibition of FAK

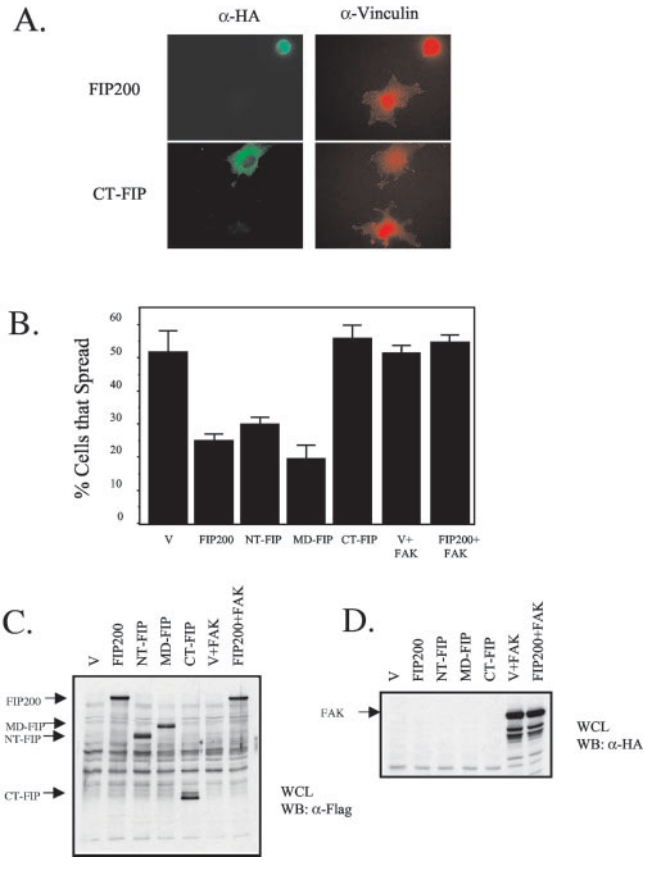

Figure 5. Inhibition of cell spreading by FIP200. (A) NIH3T3 cells were transfected with expression vectors encoding FIP200 or CT-FIP, as indicated. One day after transfection, cells were trypsinized and replated on FN (10 μg/ml) for 45 min. Cells were fixed and processed for immunostaining with anti-HA to detect the positively transfected cells (green) and with antivinculin to visualize all cells (red). (B-D) NIH3T3 cells were transfected with vectors encoding FIP200, its segments, or empty vector control, and plasmid encoding a HA-tagged FAK (in some experiments), along with a plasmid encoding β-Gal, as indicated. One day after transfection, cells were trypsinized and replated on FN (10 μg/ml) for 45 min. The cells were then fixed, and β-Gal assays were performed to identify the positively transfected cells. The mean ± SE of percentage of spread cells from three independent experiments are shown (B). Aliquots of whole cell lysates (WCL) were also analyzed directly by Western blotting using anti-Flag to detect FIP200 and its fragments (C) or anti-HA to detect FAK (D).

kinase and cellular activities in vivo (see Figures 3–7). Therefore, we designed a FAK construct, designated KD^{KR}, which contains only the kinase domain of FAK (residues 403–672) with the kinase-defective mutation (K454 to R). Overexpression of KD^{KR} should titrate out the FIP200 functional binding sites for the FAK kinase domain, thus relieving its inhibition of FAK. The kinase-defective mutant was used instead of the wild-type kinase domain construct to minimize potential effects of expressing this domain (as a kinase) other than its competing with endogenous FAK kinase domain binding to FIP200. This mutation did not affect its binding to FIP200 as KD^{KR} bound to FIP200 as efficiently as its wild-type kinase domain counterpart (S. Abbi and J. Guan, unpublished data).

We first examined the effects of KD^{KR} on FAK phosphorylation during cell adhesion. Consistent with FIP200 being an inhibitor for FAK, overexpression of KD^{KR} led to an increased tyrosine phosphorylation of FAK in cells plated on PLL in comparison with cells transfected with a control plasmid (Figure 8A). The specificity of KD^{KR} to affect FIP200/FAK interaction was supported by its lack of an effect on tyrosine phosphorylation of Pyk2 with or without stimulation by sorbitol (Figure 8B). We then examined the effects of KD^{KR} on FAK-dependent cell cycle progression by measuring BrdU incorporation of cells plated on FN or PLL. Figure 8C shows that a significant fraction (~50%) of cells plated on FN progressed to the S-phase of cell cycle under the experimental conditions, whereas only a small portion (~10%) of cells plated on PLL entered the S-phase. In contrast, overexpression of KD^{KR} led to a partial rescue of the reduced cell cycle progression on PLL (~30%). These results suggest that disruption of FAK inhibition by FIP200 could lead to an increased FAK phosphorylation as well as a partial restoration of cell cycle progression in the absence of cell adhesion to FN. It is likely that additional signals from FN other than FAK phosphorylation are necessary for a full restoration of cell cycle progression. Nevertheless, these data provide further support for FIP200 as a protein inhibitor for FAK.

DISCUSSION

FIP200 is a novel cellular protein that was recently found to interact with the FAK-related kinase, Pyk2, using the yeast two-hybrid screen (Ueda *et al.*, 2000). Furthermore, FIP200 could inhibit the kinase and cellular activities of Pyk2 by binding to its kinase domain directly. In contrast to the restricted expression pattern of Pyk2 (Avraham *et al.*, 1995; Lev *et al.*, 1995), FIP200 is widely expressed in many tissues and cell lines (Nagase *et al.*, 1996; Ueda *et al.*, 2000), suggesting that it may play important functions in some fundamental cellular processes involving FAK. In this report, we provide evidence demonstrating that FIP200 is a novel protein inhibitor for FAK. FAK has been well documented to play an important role in signal transduction by integrins. Recent studies have identified multiple signaling molecules that interact with FAK and mediate its downstream pathways in the regulation of cellular functions (Parsons, 1996; Cary and Guan, 1999; Schlaepfer *et al.*, 1999). To our knowledge, however, FIP200 is the first reported protein inhibitor for FAK that functions by directly bind to its kinase domain thus inhibiting its kinase and cellular activities.

Together with our previous report (Ueda *et al.*, 2000), these results suggest that FIP200 could function as a protein inhibitor for both members of FAK family tyrosine kinases, FAK and Pyk2. Furthermore, inhibition of FAK and Pyk2 by FIP200 may be mediated by similar mechanisms because both involve binding of FIP200 to the catalytic domains of the kinases. In contrast, we could not detect binding of FIP200 to another tyrosine kinase Src (S. Abbi, H. Ueda, and J. Guan, unpublished data), suggesting specificity of FIP200 toward FAK family kinases. Both FAK and Pyk2 have been shown to associate with Src family kinases upon their activation (Chan *et al.*, 1994; Cobb *et al.*, 1994; Schaller *et al.*, 1994;

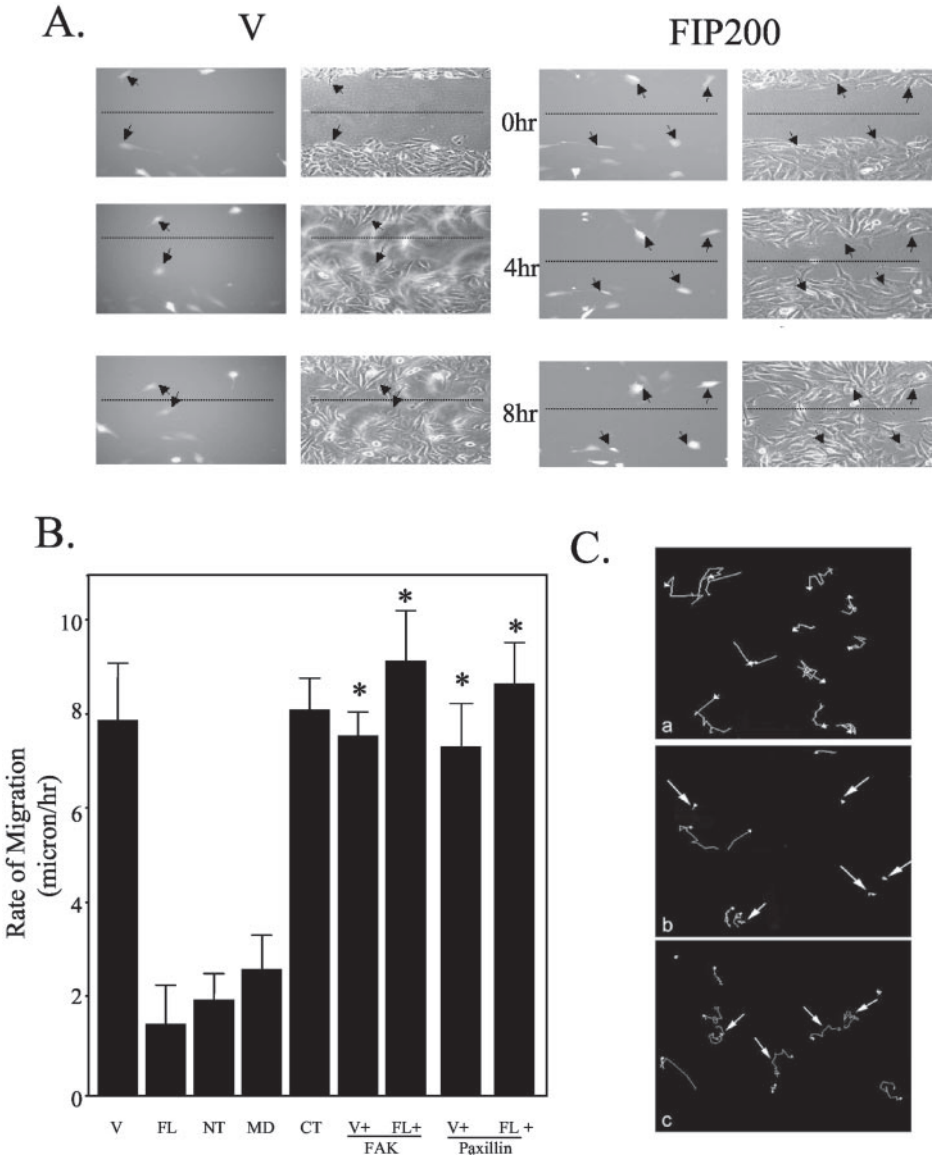

Figure 6. Inhibition of cell migration by FIP200. (A and B) NIH3T3 cells grown on FN (10 μg/ml) were transfected with FIP200, its segments, or empty vector control, with vectors encoding FAK or paxillin in some experiments, along with a plasmid encoding GFP in 7:1 ratio, as indicated. One day after transfection, the cell monolayer was wounded with a p10 tip, incubated at 37°C, and images were captured at 2-h intervals until 8 h. Images from representative experiments are shown in A. The rate of migration was measured by quantifying the total distance that the positively transfected cells (GFP^+) moved from the edge of the wound toward the center of the wound in 8 h. (B) mean ± SE of the rate of migration from three independent experiments. *p = 0.48, 0,76, 0.32, and 0.89 for samples from left to right, in comparison with vector alone transfected cells. (C) Motility of cells on FN (5 μg/ml) were also assayed using OMAware based on time-lapse video microscopy as described in "Materials and Methods." Representative field of cell tracks of control untransfected cells (a), cells transfected with expression vector encoding FIP200 (b), and cells transfected with both vectors encoding FIP200 and FAK (c) are shown. The arrows denote positively transfected cells. The untransfected cells in the same field serve as internal controls.

Xing *et al*., 1994; Lev *et al*., 1995). However, we showed previously that FIP200 inhibited the kinase activity of Pyk2 from SYF cells (deficient in Src, Yes, and Fyn expression) to the same extent as Pyk2 from wild-type control cells (Ueda *et al*., 2000). Similar results were obtained for FAK isolated from SYF or control cells (S. Abbi and J. Guan, unpublished data). These results supported that FIP200 inhibited the kinase activity of FAK and Pyk2 directly, with little effect on the associated Src family kinases.

Despite these similarities of FIP200 inhibition of FAK and Pyk2, different segments of FIP200 are involved in its interaction with the catalytic domains of these two kinases. Although CT-FIP, but not NT-FIP or MD-FIP, bound to Pyk2 kinase domain (Ueda *et al*., 2000; see Figure 2C), we found here that both NT-FIP and MD-FIP associated with FAK kinase domain and inhibited its kinase and cellular activities. Furthermore, CT-FIP bound to the N-terminal domain of FAK, reduced FAK kinase activity in vitro only when used at high concentrations (but no effect at lower concentrations), and did not inhibit FAK functions in vivo. These results suggest that different residues of FIP200 are involved in its binding to FAK and Pyk2 despite their homologous kinase domains. Indeed, in spite of the homology between FAK and Pyk2, they are activated by different signals within the cell and play different functional roles in vivo. In addition to common binding partners, there are also proteins that bind to one but not the other. For example, NIRS (mammalian homolog of *Drosophila* retinal degradation B; Lev *et al*., 1999) binds to Pyk2 but not FAK. Similarly, talin can differentiate between the C-terminal domains of FAK and Pyk2, which are 39% identical, and binds only to FAK (Zheng *et al*., 1998). Recently, a novel protein, PSGAP, has been discovered that can bind both Pyk2 and FAK in vitro, but plays a role only in Pyk2-mediated signaling (Ren *et al*., 2001). Thus,

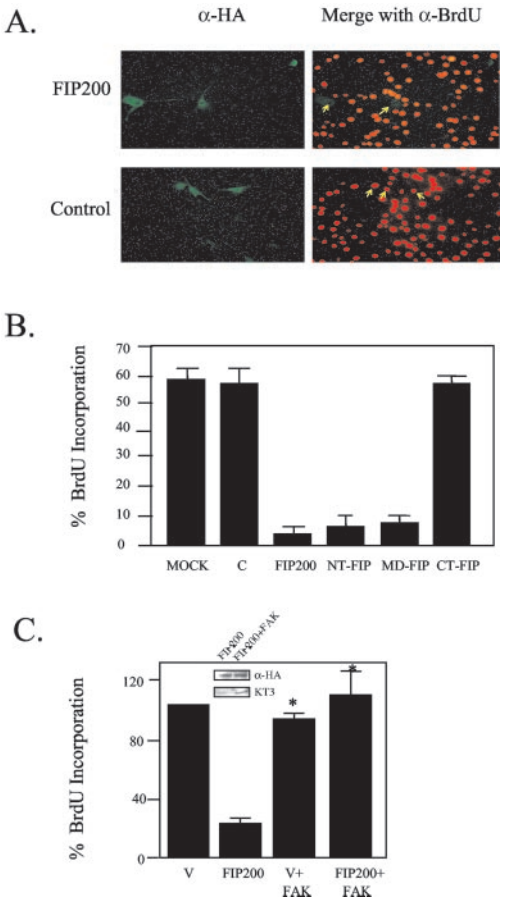

Figure 7. Regulation of cell cycle progression by FIP200. (A and B) NIH3T3 cells were transfected with expression vectors encoding HA-FIP200 or its segments, or an irrelevant control protein (C) or were mock transfected, as indicated. They were then analyzed for BrdU incorporation as described in "Materials and Methods." (A) Representative fields for cells transfected with HA-FIP200 or the control. Immunostaining with anti-HA identifies positively transfected cells (green), and staining with anti-BrdU shows cells with new DNA synthesis (red). (B) The mean ± SE of three independent experiments of the percentage of $BrdU^+$/positively transfected cells as determined by analyzing at least 80 positively transfected cells for each transfection in multiple fields. (C) NIH3T3 cells were cotransfected with an expression vector encoding FAK and that encoding HA-FIP200 or empty vector control (V), as indicated. A plasmid encoding β-Gal was also included. They were then analyzed for BrdU incorporation as described in "Materials and Methods." The positively transfected cells were identified by immunostaining with anti-β-Gal. The percentage of $BrdU^+/\beta\text{-}Gal^+$ cells was determined by analyzing 40–50 $\beta\text{-}Gal^+$ cells for each transfection in multiple fields. The percentage of BrdU-positive cells was normalized to the vector control of 100%. The results show mean ± SE of three independent experiments. *p = 0.18 and 0.59 are values for empty vector plus FAK transfection and HA-FIP200 plus FAK transfection, respectively, in comparison with value from empty vector alone transfection. Inset shows similar expression levels of FIP200 (α-HA blot) with or without cotransfection of FAK (KT3 blot).

it seems that these proteins are able to utilize the subtle differences in Pyk2 and FAK to mediate differential interactions and functions. The differential binding of FIP200 to FAK and Pyk2 also raised the interesting possibility that FIP200 may interact with both kinases and coordinate their signaling functions under certain conditions. Experiments are in progress to define the sequence motifs involved in FIP200 interaction with FAK as well as Pyk2 that should provide further insights into the molecular mechanisms and possible relationship of the interactions.

Integrin signaling through FAK has been shown to regulate a variety of cellular functions, including cell spreading, migration, and cell cycle progression (Clark and Brugge, 1995; Schwartz *et al.*, 1995; Parsons, 1996; Cary and Guan, 1999; Schlaepfer *et al.*, 1999). Consistent with its being a protein inhibitor for FAK, overexpression of FIP200 or its fragments in fibroblasts inhibited these cellular functions. Several lines of evidence suggest that FIP200 affects these cellular functions through its inhibition of FAK, although we cannot completely exclude the possible involvement of other mechanisms. First, inhibition of cell spreading, migration, and cell cycle progression by FIP200 was completely rescued by coexpression of FAK. Second, inhibition of these cellular activities by FIP200 segments correlated with their abilities to bind FAK kinase domain and inhibit its biochemical activities (as measured by autophosphorylation) in vivo (Figure 3B). It is also interesting to note that expression of either the full-length FIP200 (Figure 1E, bottom panels) or its fragments (S. Abbi and J. Guan, unpublished data) did not affect FAK localization in focal contacts, suggesting that FIP200 did not inhibit these cellular activities by altering FAK localization. Third, expression of NT-FIP reduced tyrosine phosphorylation of several FAK downstream targets, including paxillin and Shc (Figure 4), which have been shown to play a role in these cellular functions. Fourth, FIP200 did not inhibit migration of $FAK^{-/-}$ cells on FN (S. Abbi, H. Ueda, and J. Guan, unpublished data), suggesting that its effect on cellular functions is specifically through its interaction with FAK. Also, disruption of functional interaction of endogenous FIP200/FAK with a FAK kinase domain (with kinase-defective mutation) construct increased FAK phosphorylation and partially restored cell cycle progression for cells plated on PLL (Figure 8). The specificity of this construct is supported by a lack of effect on stimulation of Pyk2 activation (Figure 8B). It is also supported by the fact that no other proteins are known to interact with this region of FAK (residues 403–672, which exclude FAK motifs such as Y397 or P712/715), thus potentially be affected nonspecifically. It is a kinase-dead version, therefore the potential nonspecific effect is minimized here also. Finally, it only affected FAK phosphorylation and BrdU incorporation for cells plated on PLL (when there is FIP200 association with FAK; see Figure 1) but not for cells plated on FN (when there is minimal FIP200/FAK complex; see Figure 1). If it enhanced BrdU incorporation by other mechanisms, one would expect it to have an effect under both conditions (e.g., cotransfection of v-Src would lead to enhanced FAK phosphorylation for cells on PLL and FN; Guan and Shalloway, 1992). Taken together, these data indicate that FIP200 also functions as an inhibitor for FAK in appropriate cellular contexts.

Based on these results, we propose the following working hypothesis for the role of FIP200 interaction with FAK in integrin-mediated cell adhesion and signaling (Figure 9). In untransfected control cells (Figure 9, A, left), some FIP200

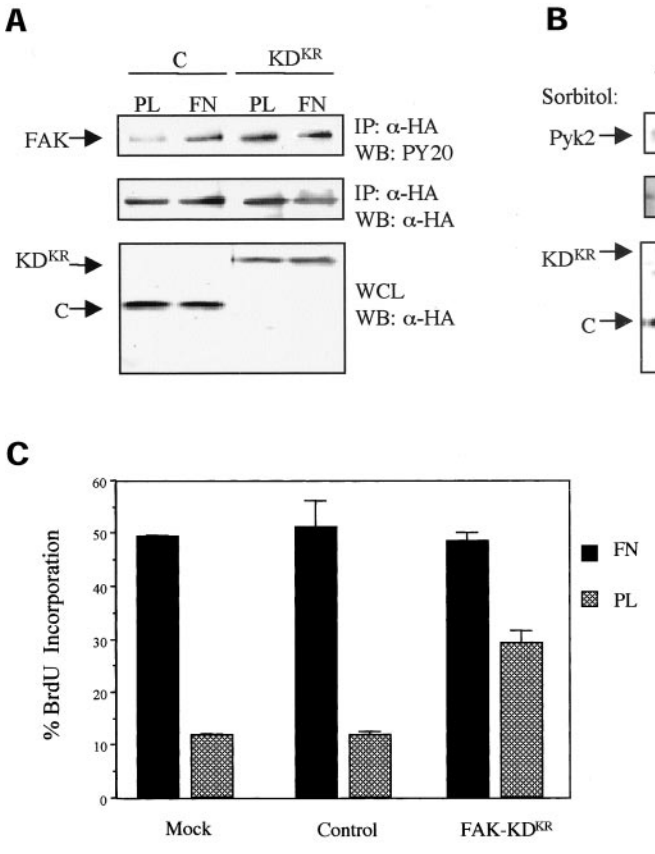

Figure 8. Disruption of endogenous FIP200 interaction with FAK. (A and B) NIH3T3 cells were cotransfected with plasmid encoding HA-tagged KD^{KR} or an irrelevant control protein (Grb7 SH2 domain, designated as C) and plasmids encoding HA-FAK or HA-Pyk2, as indicated. One day after transfection, cells were trypsinized and replated on PLL (0.1 mg/ml) or FN (10 μg/ml; A) or serum starved and treated with or without sorbitol (400 mM, 10 min; B), as indicated. Cell lysates were then immunoprecipitated with anti-HA or anti-Pyk2 and were western blotted with PY20 (top panel) or anti-HA (middle panel). Whole cell lysates (WCL) were also blotted with anti-HA (bottom panel) to show levels of transfected KD^{KR} and control protein (C). (C) NIH3T3 cells were transfected with plasmid encoding HA-tagged KD^{KR} or an irrelevant control protein, or mock transfected, as indicated. They were then analyzed for BrdU incorporation on both FN (10 μg/ml) and PLL (0.1 mg/ml) as described in "Materials and Methods." The percentage of $BrdU^+$/positively transfected cells was calculated and normalized to that of untransfected cells in each experiment. The mean ± SE are shown for data from three independent experiments.

and FAK is complexed under suspended conditions. On cell adhesion and integrin binding to ligands, FIP200 is dissociated from FAK. This release from a negative inhibitor may contribute to FAK activation and phosphorylation in cell adhesion, which trigger downstream signaling pathways in various cellular functions such as cell migration and proliferation. Overexpression of FIP200 in these cells (Figure 9, B, Right) drives the equilibrium toward more association of FIP200 with FAK (even in adherent cells), thus leading to inhibition of FAK signaling and function. These hypotheses are consistent with results in Figure 1 and other observations (Clark and Brugge, 1995; Schwartz *et al.*, 1995; Parsons, 1996; Cary and Guan, 1999; Schlaepfer *et al.*, 1999). Although this model implies some role for FIP200 in the regulation of FAK activation by integrins, it is important to note that other factors are also likely to be critical in the activation of FAK by integrins or other receptors.

One potential concern for our proposed role of FIP200 as a protein inhibitor for FAK is that the data are largely based on the overexpression of FIP200 or its fragments. It is possible that proteins of components of positive active complexes might act as dominant inhibitors when overexpressed (e.g., overexpression of the p85 subunit inhibits the PI3K function of the p85/p110 complex). In this study, however, the overexpression studies are supported by data from other and complementary approaches. These include the association and regulation of endogenous proteins (Figure 1), in vitro studies using purified proteins (Figures 2 and 3A), and expression of an FAK segment that disrupts the functional interaction of FIP200 with FAK (Figure 8). Given the consistent results from these other approaches, it is very unlikely that the endogenous FIP200 functions as a part of positive FAK complex.

It was interesting that FIP200 inhibited FAK-mediated activation of paxillin and Shc, whereas it had no effect on p130cas and Grb7 phosphorylation. It is possible that there is difference in the threshold activity of FAK required to activate its various substrate, and although the inhibition of FAK activity by FIP200 was sufficient to block its activation of paxillin and Shc, it did not effect the activation of other downstream targets. It is also possible that there are separate complexes of FAK with its various substrates, and their interaction with FIP200 is differentially regulated within the cell. In any case, these data suggest that inhibition of FAK-mediated tyrosine phosphorylation of paxillin and/or Shc by FIP200 is at least partially responsible for the inhibition of various cellular activities by FIP200. Interestingly, inhibition of cell spreading by FRNK correlated with a decreased tyrosine phosphorylation of paxillin (Richardson and Parsons, 1996; Richardson *et al.*, 1997). Furthermore, it was reported recently that tyrosine phosphorylation of paxillin and its association with Crk stimulated migration of a tumor cell line NBT-II on collagen (Petit *et al.*, 2000). Also, the phosphatase PP2A that dephosphorylates paxillin negatively regulates cell cycle progression and cell motility (Wera and Hemmings, 1995; Ito *et al.*, 2000). Consistent with a role for

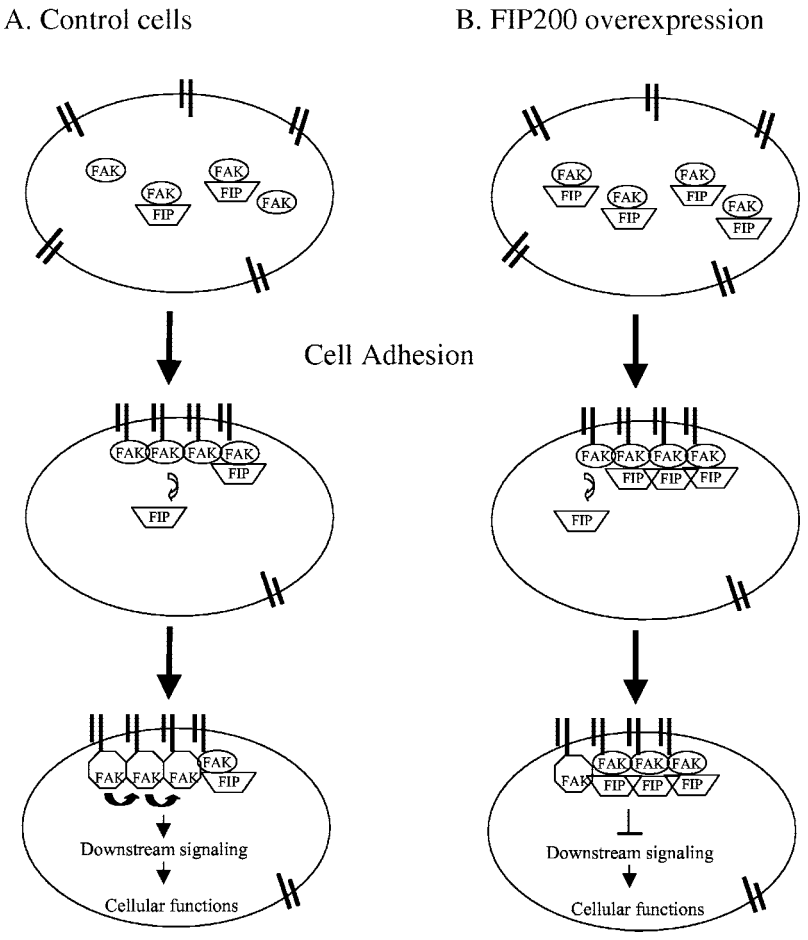

Figure 9. A working hypothesis of FIP200 interaction with FAK during cell adhesion.

paxillin in cell motility, we also observed that overexpression of paxillin rescued FIP200 inhibition of cell migration (Figure 6B). Further studies will be necessary to clarify the roles of various FAK-downstream targets in the regulation of cellular activities by FIP200.

Previous studies have shown a number of protein tyrosine phosphatases that inhibit FAK signaling by dephosphorylation of FAK (Arregui *et al.*, 1998; Tamura *et al.*, 1998; Yu *et al.*, 1998; Angers-Loustau *et al.*, 1999; Manes *et al.*, 1999; Miao *et al.*, 2000;). However, all these inhibitory events required the enzymatic activities of the phosphatases. In contrast, FIP200 inhibited FAK by binding to its kinase domain, which offers the potential opportunity to derive small peptide inhibitors for FAK. It is interesting that two FIP200 segments (NT-FIP and MD-FIP) could both inhibit FAK by apparently similar mechanisms. There are several regions of high homology (~30% identity) between NT-FIP and MD-FIP. Future studies will be necessary to determine whether these common regions play a role in FIP200 interaction with FAK. The possible generation of small peptides or their derivatives as inhibitors for FAK is also an exciting future avenue of research, especially because activation of FAK has been implicated in diseases such as cancer metastasis (Weiner *et al.*, 1993; Owens *et al.*, 1995).

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